Structural Characterization and Detection of Kale Flavonoids by Electrospray Ionization Mass Spectrometry

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Sensitive and precise analytical methods are needed for flavonols, a subclass of flavonoids that has strong antioxidant activity. We report an improved method for identifying the predominant flavonols, quercetin and kaempferol, by collisionally activated dissociation (CAD) and quantifying them by high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) in the selected ion monitoring mode. Practical applications of the method were demonstrated using several kale and biological samples. Two commercial kale samples were found to have 77 or 244 ppm quercetin and 235 or 347 ppm kaempferol (ppm = μg of quercetin/g of kale or μg of kaempferol/g of kale by fresh weight, 5−15% relative standard deviation). Blanching was found to reduce the flavonols to ∼60% of the levels found in the unblanched kale. Isotopically labeled kale (cultivar Vates) grown in a greenhouse under an atmosphere of $^{13}CO_2$ was found to have much lower flavonol levels. UV-A and UV-B supplementation during kale growth in the greenhouse was found to enhance both quercetin and kaempferol levels in Vates kale. The UV-B-supplemented kale not only produced more flavonols but the quercetin-to-kaempferol ratio was also higher than the UV-A-supplemented or the nonsupplemented kale. Recovery of flavonols from kale was ∼60% based on spike and recovery trials with rutin, a glycoside of quercetin. Recovery of flavonols from biological samples spiked with rutin ranged from 96% for urine to 70% for plasma. Compared to UV detection, ESI-MS in the deprotonation mode provided lower detection limits, and both higher sensitivity and selectivity, in addition to structural characterization of the kale flavonoids by CAD.

Flavonoids are a large group of phytochemicals ubiquitously found in fruits and vegetables. They have the general structure of a 15-carbon skeleton, which consists of two phenyl rings and a heterocyclic ring.[1,2] According to the differences in functional groups and their relative positions of the 15-carbon skeleton (aglycons), flavonoids are classified into several subgroups including the following: flavone, flavanone, flavonol, isoflavonoid, anthocyanidin, and chalcone. In plants, most flavonoids are conjugated to a carbohydrate moiety, and thousands of flavonoids have been known with individual differences arising in the various hydroxylation, methoxylation, glycosylation, and acylation patterns.[3]

Flavonols, a subclass of flavonoids, have in common a 3-OH group on the pyrone ring. In the United States, consumption of flavonols is estimated to be 20−23 mg/day,[4] and quercetin, kaempferol, and myricetin are the most abundant dietary flavonols. Common foods, predominantly onion, apple, and tea, are the major food sources of dietary flavonols in Danish[5] and American[4] diets; and kale, capers, cocoa, cranberry, and some peppers are other rich sources.[6−8]

Flavonoids are well known for their antioxidant abilities and hold promise for preventing age-related diseases including heart disease and cancer.[1,9−10] For example, consumption of the flavonol-rich foods onion, apple, and tea was shown to be inversely related to coronary heart disease mortality,[11] albeit the finding has not been consistently demonstrated.[12] Most structure−activity relationship studies have focused on the aglycons, whose multiple hydroxyl groups and the C2−C3 double bond are thought to play

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a critical role in their chemopreventive activities.[13] Though flavonoids have attracted a lot of attention due to their purported health benefits, little is known about their absorption, bioavailability, and metabolism. According to several recent studies, flavonoids have very low bioavailability and they are likely to be conjugated to glucuronide and sulfate groups after consumption.[14−21] After pure quercetin (including glycosides) or quercetin- and kaempferol- rich food was consumed, quercetin[14−18,20] or both quercetin and kaempferol[19,21] were detected in plasma even though the amounts were low (in the range of 7.3−7700 nM quercetin and 0−500 nM kaempferol), and there were large variations among the different reports. Quercetin[22,23] or quercetin and kaempferol[21,24−27] were also detectable in urine after flavonoid consumption. Either acid or enzyme hydrolysis was usually used to convert flavonoid metabolites (usually glucuronides and sulfates) or glycosides to their aglycon forms before detection. Intact flavonoid glycosides in vivo were only occasionally detected,[19−20,28−29] which may be due to the prevalent use of hydrolysis, lack of sensitive detection methods, or the common practice of freezing or otherwise delaying analysis.[30] Further systematic and well-controlled studies are needed before the bioavailability and the in vivo chemopreventive properties of the flavonoids (including their metabolites) can be fully understood.

UV absorption has been commonly used to detect or quantify flavonoids[8,17,18,23,26,28,29,31−34] with limits of detection, when reported,
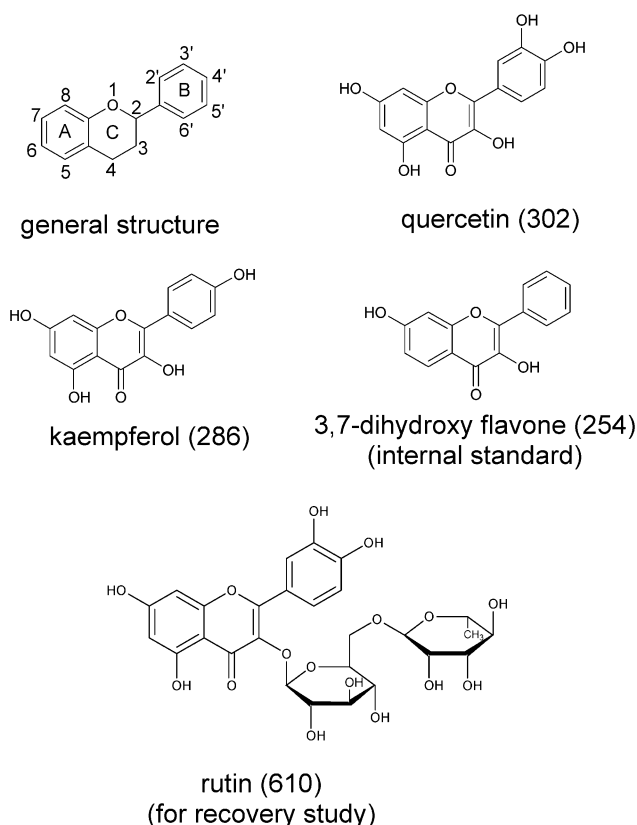

Figure 1. Flavonoid structures (molecular mass in Da).

falling in the range of 0.1−10 ng.[17,32] Hollman et al.[35] developed a sensitive fluorescence-based detection method for flavonoids that involved postcolumn chelation with aluminum ions and that was used subsequently in other studies.[21−23,27] The limits of detection for this method were reported as 0.15 and 0.05 ng/mL for quercetin and kaempferol, respectively, in plasma; however, this fluorescence-based method was limited to flavonols, mainly as aglycons, because only compounds with a 4-keto oxygen and a free 3-hydroxyl group chelated the Al^{3+} ion to form the fluorescent complexes.[32] Radioactivity counting[20] and electrochemical[14−16,33] methods yielded lower limits for some of the flavonoids but without providing any structural information, which is critical for metabolite identification. In fact, both GC/MS[15,24] and LC/MS[17−20,25,29,33,34] have been successfully applied for metabolite identification. Among the only three studies that used LC/MS for quantification of quercetin[17] or both quercetin and kaempferol,[19,25] one achieved a detection limit of 2 ng in plasma (similar to LC−UV) using positive electrospray ionization[17] while the second quantified quercetin and kaempferol metabolites separately in plasma.[19] The last study[25] achieved a limit of quantification in urine in the same order of magnitude as that of Hollman et al.[35]

Kale is a vegetable that is easy to grow and has been reported to have high levels of flavonoids with the two most common aglycons, quercetin and kaempferol[8,31−32] (see Figure 1 for structures of quercetin and kaempferol). Both of these flavonoids have shown antimutagenic and anticarcinogenic effects. It is therefore our interest to establish a protocol to extract and quantify these flavonoids in kale, urine, and plasma. Since flavonoids are

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ubiquitous in plants, extremely careful experimental design is demanded to avoid the interference of flavonoids from other dietary sources during human feeding studies aimed to track the metabolism and bioavailability of specific flavonoids from specific food sources. Eliminating all other sources of flavonoids from the diet can be prohibitively restrictive for practical studies. In addition, it becomes increasingly complex to monitor specific flavonoids if they become conjugated or are metabolized to other compounds in the body. Metabolism and bioavailability studies are unambiguous if one has access to isotopically labeled flavonoids in food sources, thus allowing identification and quantification of the flavonoids, their conjugates, and their metabolites based on tracking the isotopically tagged compounds. This can be conveniently done by monitoring the mass shifts of the isotopically labeled compounds by mass spectrometry. Isotopic labeling as demonstrated in this work is very useful in elucidating fragmentation/metabolism pathways and can be used for unambiguous peak assignments. However, isotopically labeled compounds are not always readily available.

In the present study, the natural biosynthetic abilities of the kale plant are used to our advantage. Kale is particularly attractive as a food source for studies of bioavailability and metabolism of quercetin and kaempferol flavonoids in humans because it can be grown rapidly under controlled conditions in growth chambers with a $^{13}CO_2$ atmosphere. Kale (cultivar Vates) was grown in a Plexiglas growth chamber with $^{13}CO_2$ as the only carbon source to produce ^{13}C-labeled flavonoids. The purpose of this study was to develop a rigorous and sensitive method for the analysis of flavonols and to apply the method to analysis of a food (kale) and biological samples (plasma and urine). In doing so, high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) in the deprotonation mode is used for quantitation of quercetin and kaempferol in urine, plasma, and kale for the current study. As a direct parallel comparison to the commonly used UV detection method, UV absorption is also monitored after HPLC separation of the flavonols. UV radiation and blanching are identified as determinants of quercetin and kaempferol concentrations in kale and protein binding as a factor in limiting recovery of flavonols from plasma.

EXPERIMENTAL SECTION

Chemicals and Reagents. Quercetin dihydrate and rutin (Figure 1) were purchased from Sigma (St. Louis, MO). Kaempferol and 3,7-dihydroxyflavone (Figure 1) were purchased from Indofine (Somerville, NJ). All the above flavonoids were used without further purification. Hydrochloric acid was purchased from Millinckrodt, Inc. (Paris, KY). Formic acid and acetic acid were purchased from EM Science (Gibbstown, NJ). Methanol, acetonitrile, acetone, chloroform, and water were all HPLC grade. Rat plasma was purchased from Pel-Freez Biologicals (Rogers, AR) and used after thawing. Urine was collected from a volunteer consuming a low-flavonoid diet and after overnight fast and used immediately.

Quercetin, kaempferol, 3,7-dihydroxyflavone, and rutin were all dissolved in methanol to $\sim 1 \times 10^{-3}$ M stock solutions.

Growth of Kale and Lighting Conditions. A complete description of the method to produce ^{13}C kale can be found in Kurilich et al.[36] In brief, ~ 80 plants (*Brassica oleracea acephala* cultivar Vates) were labeled uniformly with ^{13}C during continuous growth in an atmosphere containing $^{13}CO_2$ (99% mol of ^{13}C; Isotec Inc., Miamisburg, OH) starting ~ 8 days after sowing. An infrared gas analyzer (IRGA, model WMA-3, PP Systems, Haverhill, MA) calibrated using $^{13}CO_2$ (IRGA is much less sensitive to $^{13}CO_2$ than normal $^{12}CO_2$) controlled the level of $^{13}CO_2$ at 400 ppm. The labeling apparatus consisted of an airtight box (total volume 676 L) constructed from clear acrylic (Plexiglas G, Rohm and Haas, Wilmington, DE). Air temperature was maintained at 24−25 °C, and humidity was controlled at $\sim 90\%$. Plants were subirrigated 1−2 times daily with a complete mineral nutrient solution. A 50:50 mix of 400-W metal halide and high-pressure sodium lamps (Sylvania-GTE, Danvers, CT) provided continuous photosynthetically active radiation (PAR, 400−700 nm), accelerating plant growth and avoiding the need to recover respired $^{13}CO_2$, which would become necessary (given the high cost of the isotope) if the kale required a light−dark cycle. As a result, daily PAR integrals (11−15 MJ m^{-2}) inside the enclosure at plant canopy height were $\sim 25\%$ greater than maximum average values outdoors. Labeling was terminated at canopy closure, which coincided with a reduced rate of increase of CO_2 consumption. Plants were rapidly harvested in dim light and stored on ice in sealed plastic bags. Leaf material was cleaned, weighed, blanched, chopped into pieces (~ 7 cm^2), mixed for batch uniformity, and then frozen at −80 °C until prepared for consumption.

In other experiments, kale (cultivar Vates) was grown in a greenhouse at Beltsville, MD, between March 17 and April 21, 2003, under natural daylengths (~ 12 h increasing to 13.5 h). Plants received ~ 3 MJ m^{-2} average daily PAR (i.e., $\sim 54\%$ of ambient), which included up to 12 h/day supplemental light from high-pressure sodium lamps (24 W m^{-2}). Day/night greenhouse temperatures were set for 26/21 °C with a daily average of 23 °C (± 1 °C standard deviation). Supplemental UV radiation was initiated when the first true leaves unfolded (March 25). Fluorescent sunlamps (UVB-313, Q-Panel, Cleveland, OH) filtered through cellulose diacetate (0.08 mm; Cadillac Plastics, Baltimore, MD) were suspended over the plant bed to simulate expected biologically effective UV-B radiation (290−315 nm) for the summer solstice at Beltsville (data not shown). Since the lamps also emitted UV-A (315−400 nm) and low levels of visible radiation, a UV-A control was set up in which plants were exposed to longer wavelength radiation from a matched set of lamps wrapped with polyester (0.13 mm; Cadillac Plastics) that blocked the shorter wavelength UV-B component. Both UV-B- and UV-A-treated plants received similar amounts of solar UV-A transmitted through greenhouse glazing ($\sim 315−400$ nm). Consequently, separate greenhouse controls were established without UV lamps and shielded from wavelengths shorter than ~ 350 nm by an acrylic sheet (similar to conditions for isotopically labeled kale). Plants were harvested as described above.

Extraction of Flavonols from Kale. The extraction procedure was modified from the Giusti and Wrolstad procedure,[37] which was originally developed for anthocyanins, another subclass of flavonoids (Figure 1). Briefly, about 5−20 g of fresh kale leaves (either nonlabeled or labeled) were blended to powder under

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liquid nitrogen. About 1−3 g of the kale powder was weighed into centrifuge tubes. Samples were vortex-mixed with 10 mL of acetone, centrifuged, and the supernatant collected. The samples were re-extracted with 10 mL of 30% aqueous acetone, and the supernatants were combined and mixed with two volumes of chloroform. The aqueous phase was collected, and residual organic solvents were allowed to evaporate before filtration. The yellowish clear solution was used for acid hydrolysis, which converted the native flavonoid glycosides to their corresponding aglycons.[38] Five milliliters of 2 N HCl in 50% aqueous methanol was mixed with 1 mL of the above kale extract (50% aqueous methanol was found to give higher recoveries than pure water or methanol during preliminary studies). The samples were hydrolyzed for 2 h in a boiling water bath.

The extracts were subjected to solid-phase extraction before separation by HPLC. A kale extract was loaded onto a C_{18} cartridge (100 mg, Waters, Milford, MA) after the cartridge was conditioned with methanol and water. The cartridge was washed with 3% aqueous methanol following which the kale flavonols were eluted by pure methanol. The eluents were brought to dryness under nitrogen and reconstituted in methanol. 3,7-Dihydroxyflavone was added to have a final concentration of 2.0×10^{-5} M as the internal standard prior to LC/MS analysis.

Since blanched kale will be used for the human feeding studies in a future investigation, the effect of blanching on the flavonol levels was investigated. The same kale leaves were divided into two equal portions: one without and one with an additional blanching process before extraction. The second portion of the kale leaves was immersed in ∼400 mL of boiling water for 2 min and then transferred to ∼400 mL of ice/water and cooled for 2 min. The blanched kale leaves were blotted dry. The rest of the extraction procedure was the same as used for the first portion of the kale leaves. Acid hydrolysis and solid-phase extraction were also performed on the water used for blanching. Both blanched and nonblanched kale samples as well as the blanched water were analyzed by LC/MS. The quercetin and kaempferol levels were then compared for the flavonol losses due to the blanching process.

Recoveries of Flavonols from Kale and Biological Samples. To access flavonol recovery from kale, rutin, a quercetin glycoside, was spiked to the kale powder (of the same kale sample) at 50 and 100% kale flavonol levels (calculated from a previous LC/MS analysis) immediately after the kale powder was weighed into the centrifuge tubes. From the second LC/MS analysis, the recovery of rutin as its aglycon form (quercetin) was calculated by dividing the calculated rutin amounts in the originally spiked solutions by the actual spiked amounts. Every kale sample including the ones for the recovery studies was extracted and analyzed at least in duplicate.

Rutin was spiked in raw urine or rat plasma at four different concentration levels within the linear range of the calibration curve (8.0×10^{-7}, 4.0×10^{-6}, 2.0×10^{-5}, and 1.0×10^{-4} M). 3,7-Dihydroxyflavone was also spiked at the same time to have a final concentration of 2.0×10^{-5} M. Urine and plasma blanks (without addition of rutin) were also included. The urine extraction procedure was modified from the literature[17] to incorporate an

additional centrifugation step (11 000 rpm for 15 min) to minimize interference from the urine matrix. To stabilize rutin in plasma,[14,18,28] 0.1 volume of 0.27% acetic acid was added to each sample, which was then vortexed and centrifuged at 14 000 rpm for 5 min. Protein precipitation was achieved by addition of four volumes of acetonitrile. Acid hydrolysis and solid-phase extraction procedures were the same as kale extraction.

HPLC. The flavonols were separated using a Waters Alliance 2690 system (Waters) with a Waters 486 tunable absorbance detector. A Waters Symmetry C_{18} column (2.1×50 mm, 3.5 μm) was used with a guard (2.1×10 mm, 3.5 μm). The mobile phase was composed of solvents A (0.33% formic acid) and B (acetonitrile with 0.33% formic acid). A mobile-phase gradient was used: 0−13 min, 30−100% B; 13−15 min, 100−30% B; 15−25 min, 30% B. The injection volume was 5 μL. The flow rate was 100 μL/min. The UV absorption of the flavonols was monitored at 360 nm. The HPLC eluent was introduced directly to the mass spectrometer without splitting.

Mass Spectrometry. A Thermo Finnigan LCQ Duo quadrupole ion trap mass spectrometer (San Jose, CA) was used for structural characterization and improved detection of the flavonols. Nitrogen was used as the sheath gas (20 arbitrary units). Conditions included a capillary temperature of 200 °C and a spray voltage of −4.5 kV, and the other parameters were optimized for the molecular ion of deprotonated quercetin.

For direct infusion experiments, a flavonol solution of ∼1×10^{-5} M in methanol was introduced to the LCQ at 5 μL/min by a syringe pump. The injection time was 10 ms for full scan and 50 ms for the MS^n experiments. The collision energy was applied in such a way that the relative abundance of the surviving parent ion from each activation step was ∼5−10%. For LC/MS analysis, the injection time was 100−200 ms except for the measurements at the limit of detection in which case the injection time was raised to 1000 ms. Selected ion monitoring (SIM) was used to monitor the deprotonated molecular species of quercetin, kaempferol, and 3,7-dihydroxyflavone at m/z 301, 285, and 253 (for unlabeled kale) or 316, 300, and 253 (for ^{13}C-labeled kale), respectively. The peak area ratios against the internal standard were used for quantitation through the calibration curves of quercetin and kaempferol, respectively.

RESULTS AND DISCUSSION

Structural Characterization. Due to the acidic nature of the flavonoids, deprotonation was found to be a much more effective ionization mode than protonation. The extracts from kale, urine, and plasma were first surveyed by ESI-MS via direct infusion prior to LC/MS analysis. Typical full-scan spectra of the kale extracts are shown in Figure 2. The parent ions of m/z 301 (or 316 for the labeled kale) and 285 (or 300 for the labeled kale) were isolated and activated by collisionally activated dissociation (CAD). Flavonol authenticity was checked against the CAD patterns of the corresponding standards in addition to the molecular ions and retention times obtained by LC/MS. As an example, the CAD spectrum of quercetin is shown in Figure 3A. The peak assignments were based on the MS^n experiments (data not shown) and also confirmed by the fragmentation patterns of the ^{13}C-labeled compounds from kale, in which the mass shifts observed matched those expected for the number of carbon atoms in each proposed

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A) Conventional kale
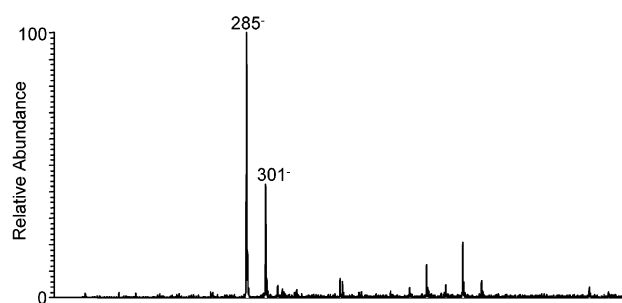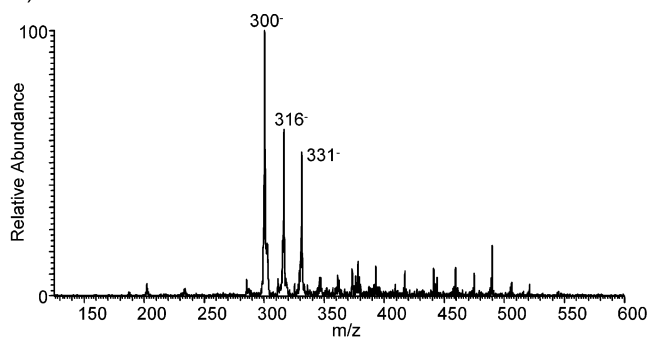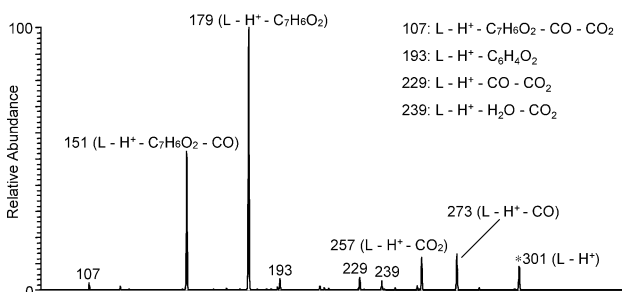

B) ^{13}C-labeled kale

Figure 2. Full ESI-mass spectra of kale extracts by direct infusion.
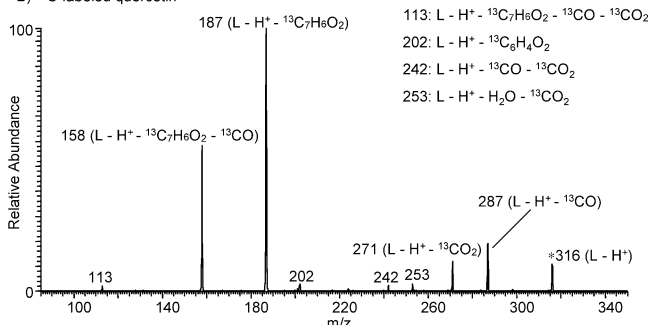
A) Regular quercetin

179 (L - H$^+$ - C$_7$H$_6$O$_2$)

107: L - H$^+$ - C$_7$H$_6$O$_2$ - CO - CO$_2$
193: L - H$^+$ - C$_6$H$_4$O$_2$
229: L - H$^+$ - CO - CO$_2$
239: L - H$^+$ - H$_2$O - CO$_2$

151 (L - H$^+$ - C$_7$H$_6$O$_2$ - CO)

273 (L - H$^+$ - CO)
257 (L - H$^+$ - CO$_2$)
*301 (L - H$^+$)
107 193 229 239

B) ^{13}C-labeled quercetin

187 (L - H$^+$ - $^{13}C_7$H$_6$O$_2$)

113: L - H$^+$ - $^{13}C_7$H$_6$O$_2$ - ^{13}CO - $^{13}CO_2$
202: L - H$^+$ - $^{13}C_6$H$_4$O$_2$
242: L - H$^+$ - ^{13}CO - $^{13}CO_2$
253: L - H$^+$ - H$_2$O - $^{13}CO_2$

158 (L - H$^+$ - $^{13}C_7$H$_6$O$_2$ - ^{13}CO)

287 (L - H$^+$ - ^{13}CO)
271 (L - H$^+$ - $^{13}CO_2$)
*316 (L - H$^+$)
113 202 242 253

Figure 3. CAD spectra of deprotonated quercetin.

fragment (Figure 3B). The fragmentation patterns were also found to agree well with the previously reported CAD mass spectra.[39]

As shown in Figure 2, the full-scan mass spectrum of the kale grown in a Plexiglas box under the $^{13}CO_2$ atmosphere gives an additional ion at m/z 331 (labeled) or 315 (nonlabeled) compared to the commercial kale samples. The dominant CAD pathway for this new unknown compound is the loss of a methyl radical, which
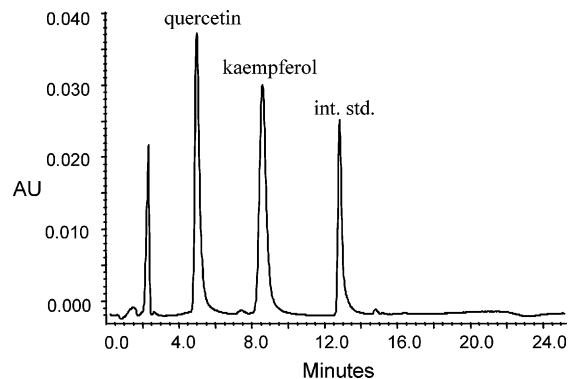

Figure 4. HPLC separation of quercetin, kaempferol, and the internal standard 3,7-dihydroxyflavone followed by UV detection.

indicates it is a methylated species.[39-41] MS^n experiments give fragment ions and a genealogy similar to that obtained for the dissociation of deprotonated rutin and isorhamnetin-3-glucoside, both of which give a quercetin-like radical ion upon sequential CAD except that the new compound also produces more fragments at the lower mass range (data not shown). In addition, the retention time of the unknown is close to that of kaempferol, eluting ~0.6 min after kaempferol. All the above facts suggest that the unknown compound is a methylated form of quercetin, although the specific site of methylation has not been pinpointed. This new methylated flavonoid is only consistently detected in the Vates kale grown in the greenhouse, not in the commercial kale.

Limits of Detection and Calibration Curves. Quercetin, kaempferol, and the internal standard 3,7-dihydroxyflavone were well separated using the above separation conditions (Figure 4). Compared to UV absorption, SIM offered higher selectivity and sensitivity (Figure 5). The detection limits were ~10 pg for quercetin and ~3 pg for kaempferol with S/N equal to or higher than 3 (with an injection volume of 5 μL). These limits were 2 or 3 orders of magnitude lower than that of the parallel UV absorption method, which were similar to literature values[17,32] or positive electrospray ionization mass spectrometry,[17] and of the same order of magnitude as that obtained by negative atmospheric pressure chemical ionization.[25] With 3,7-dihydroxyflavone as the internal standard, the calibration curves were linear from 1×10^{-7} to 3×10^{-4} M (~0.03 to 90 μg/mL, eight concentrations tested) for both quercetin ($y = 0.0556x + 0.199$, x in μg/mL, $R^2 = 0.9785$) and kaempferol ($y = 0.0635x + 0.0876$, x in μg/mL, $R^2 = 0.9972$). It should be noted that the above equations and R^2 values are shown here only as an example of typical values since they varied slightly from day to day, which is the nature of the mass spectrometric response.

Detection of the Kale Flavonoids by LC/MS. The clearly higher selectivity of SIM was evident for the kale samples (Figure 5). Although the chromatogram obtained by UV absorption detection (Figure 4) was satisfactory for the standards, the baseline for the kale samples (Figure 5A) was excessively high due to the complex matrix effect. In addition, there were several interference peaks surrounding the peaks of interest (Figure 5A), which severely affected the integration of the peaks, especially

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(40) Justesen, U. *J. Mass Spectrom.* **2001**, *36*, 169−178.

(41) Zhang, J.; Brodbelt, J. S. *J. Mass Spectrom.* **2003**, *38*, 555−572.

A. Detection by UV
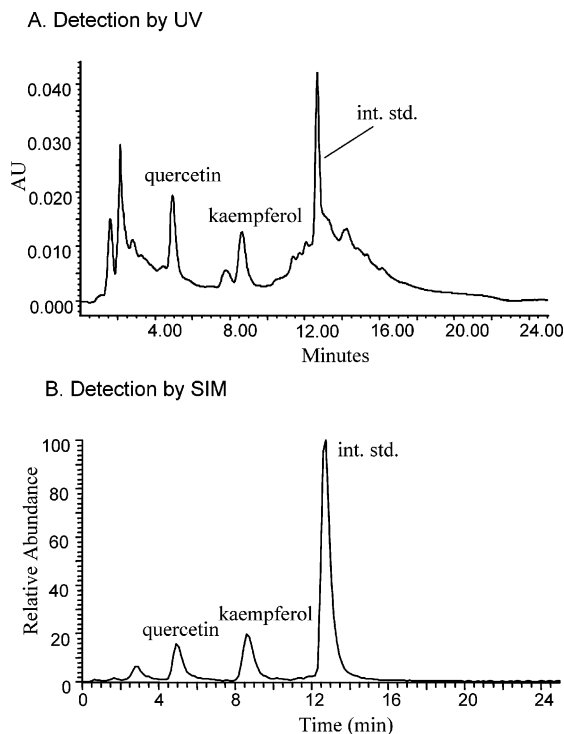

B. Detection by SIM

Figure 5. HPLC chromatograms of the flavonoids followed by UV and mass spectrometric detection in a kale sample.

for the internal standard. In contrast, there was no interference found in the kale samples by SIM (Figure 5B) because of the much higher selectivity of SIM, which was also true for the urine and plasma samples.

The kale flavonols were successfully recovered as judged by use of rutin as a model compound. The flavonols in kale (as in other vegetables and fruits) are known to exist mainly as glycosides. The addition of an aglycon internal standard, such as 3,7-dihydroxyflavone, to the kale samples would not account for either the difference in extraction efficiencies of glycosides versus aglycons or the conversion efficiency of the glycosides to the aglycons quercetin and kaempferol, which were our interest. Thus, rutin, a quercetin glycoside, was chosen as a model compound because it should reflect the recoveries for the entire extraction procedures and the acid hydrolysis more realistically than a flavonoid aglycon. Kale powder was spiked with rutin before liquid extraction so that its absolute recovery could be monitored. The recoveries of rutin (as its aglycon quercetin) at two different spiking concentrations (50 and 100% of the flavonoids in kale) were 65 and 53%, respectively.

The flavonols found in two commercial kale samples were 77 or 244 ppm quercetin and 235 or 347 ppm kaempferol (ppm = μg of quercetin/g of kale or μg of kaempferol/g of kale, fresh weight). The relative standard deviation of the duplicates was $\sim$5−15%. The previously reported level of quercetin in kale varied from 7 to 120 ppm by fresh weight.[8,31−32] Likewise, the content of kaempferol varied from 13 to 470 ppm by fresh weight.[8,31,32] All of these literature values were obtained by HPLC−UV detection. The wide range of flavonols previously reported in kale and in our samples of kale may be due to the different variety and source of kale, the extraction efficiency, and the age and storage conditions of the kale.

Effects of Blanching. Blanching is a common food preparation method in which foods are briefly suspended in a relatively large volume of boiling water and then quenched in ice water, rapidly halting enzymatic processes and preserving nutrient content. It is often used to prepare raw vegetables and to improve both palatability and nutrient bioavailability. However, flavonol glycosides present in plants are water-soluble. Since isotopically labeled kale used for human feeding studies was blanched prior to storage at −80 °C, we determined the impact of this procedure on the flavonol levels. Blanching was found to reduce the flavonols to $\sim$60% of their native values (i.e., 244 ppm quercetin and 347 ppm kaempferol without blanching vs 151 ppm quercetin and 223 ppm kaempferol with blanching). As expected, large amounts of the kale flavonols were found in the blanching water (1140 μg of quercetin and 2660 μg of kaempferol in the total of 400 mL of water used). The facts that the blanching water is rich in the flavonols and that the flavonols survive the 2-h hydrolysis in a boiling water bath indicate that the flavonols are stable even at elevated temperatures (at least up to 100 °C for 2 h). Thus, the blanching process does not degrade the flavonols but instead leaches $\sim$40% of the flavonols into the blanching water due to the high water solubilities of the flavonol glycosides. The blanching effects on flavonols have also been reported in the literature.[42,43] About 50% of quercetin was lost in sweet potato leaves after 2 min of boiling,[42] while the loss of flavonoids in broccoli florets could be up to 86% after 15 min of boiling.[43]

Analysis of Isotopically Labeled Kale. After harvesting the first crop of isotopically labeled kale, flavonol concentrations were measured and found to be $\sim$10 times lower than in the commercial kale samples (i.e., 6.8 ppm quercetin and 23 ppm kaempferol by fresh weight in the isotopically labeled kale). Although blanching could account for part of this difference, it was unclear to what extent possible differences between commercial kale (unknown genetic line) and labeled kale (cultivar Vates) or factors related to conditions of growth and storage might be involved. For example, isotopically labeled kale was raised under relatively high levels of continuous light. In addition, the acrylic chamber in which the kale was grown blocked most ultraviolet radiation at wavelengths shorter than 350 nm, whereas kale grown outdoors would have received the complete solar spectrum (wavelengths longer than $\sim$290 nm). UV and visible radiation has been widely reported to affect flavonoid levels in plants, including several Brassica species.[44−46]

Effects of Ultraviolet Radiation. To test the influence of ultraviolet radiation on the production of flavonols, Vates kale was grown in a greenhouse and supplemented with UV-A alone (315− 400 nm) or with UV-A plus UV-B (290−400 nm). Greenhouse controls were shielded from radiation lower than 350 nm. Growth conditions were similar for all treatments (Table 1). Flavonol levels in the greenhouse controls were similar to those for labeled kale after correction for blanching of the latter samples. Both UV

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Table 1. Supplemental Ultraviolet Radiation, Growth at Harvest, and Flavonol Concentration in Greenhouse Kale (Cultivar Vates)

	greenhouse control	supplement	
		UV-A	UV-B
average daily radiation, kJ m^{-2}			
UV-B, 280−315 nm	a	0.04	12.96^b
UV-A, 315−350 nm	a	15.18^c	24.60^c
UV-A, 350−400 nm	c	4.54^c	5.11^c
average fresh wt per shoot, g ^{d}	48	54	48
flavonoids, ppm (fresh wt basis)^e			
quercetin	12.6	21.1	108
kaempferol	42.0	71.6	254
quercetin/kaempferol ratio	0.30	0.29	0.43

^{a} Acrylic filter blocks these wavelengths. ^{b} More than 99.97% at wavelengths longer than 290 nm. ^{c} Solar radiation transmitted by greenhouse glazing not included. ^{d} $n = 4$. ^{e} Relative standard deviations average 5−15%.

treatments caused significant increases in flavonols (Table 1), but the effect was much greater when UV-B was included. In the presence of UV-B, kaempferol and quercetin levels increased 6- and 8.5-fold, respectively, over the greenhouse controls. As a result, the ratio of quercetin to kaempferol also increased in UV-B-irradiated kale. By comparison, this ratio was 0.7 in the commercial sample with high flavonols (∼600 ppm quercetin + kaempferol). Relative increases in quercetin may have biological significance because it has been reported that quercetin has better free radical scavenging ability than kaempferol.[13]

Urine and Plasma Extraction. Urine and plasma blanks did not have any detectable flavonols. Rutin was successfully recovered from urine as its aglycon form, quercetin. The recoveries were above 96% for all the four spiking concentrations within the linear range of the calibration curve: 8.0×10^{-7}, 4.0×10^{-6}, 2.0×10^{-5}, and 1.0×10^{-4} M. Prior reported recovery values in urine were 73 (for the solid-phase extraction step only)[24] and 88%.[22] To obtain a reproducibility that is similar to kale and urine extraction, it was necessary to acidify plasma samples prior to protein precipitation. Presumably due to the severe interference of proteins in the plasma, lower recoveries (69−74%) were found for plasma samples at the above four spiking levels, which was a little lower than the two previously reported values: ∼95% for quercetin[15] and above 90% for rutin as the native glycoside form.[28] Direct comparison of the recovery values might not be appropriate since we recovered rutin as quercetin while the other studies recovered either quercetin[15] or rutin[28] in its native form after spiking plasma directly with quercetin or rutin, respectively. In addition, our recoveries of rutin are assessed relative to the aglycon internal standard, 3,7-dihydroxyflavone, which likely has a extraction efficiency different from that of rutin, a glycoside.

CONCLUSIONS

An HPLC-ESI-MS protocol for detection of the flavonols in kale and biological samples was successfully established for the first time. The detection limits by HPLC-ESI-MS (10 pg of quercetin and 3 pg of kaempferol) were ∼100−1000 times lower than the commonly used HPLC−UV method. The selectivity of HPLC-ESI-MS was also much higher with the additional advantage of structural characterization of the flavonols. Calibration curves were linear for a wide dynamic range: 1×10^{-7}−3×10^{-4} M (∼0.03−90 μg/mL). Kale was found to have 77 or 244 ppm quercetin and 235 or 347 ppm kaempferol for two conventional samples (ppm = μg of quercetin/g of kale or μg of kaempferol/g of kale, fresh weight). During a simple blanching process, 40% of the kale flavonols were lost to the blanching water. The flavonols in the isotopically labeled, greenhouse-grown, blanched kale were ∼10 times lower than that of conventional unblanched kale. UV supplementation greatly enhanced the flavonol levels in the same genetic line of kale by factors of 6 for kaempferol and 8.5 for quercetin. The extraction efficiencies and recoveries of flavonols from kale, urine, and plasma were found in the range of 60% for kale, 96% for urine, and 70% for plasma judged from the measuring recovery of quercetin after spiking with rutin.